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Development of loop-mediated isothermal amplification (LAMP) for rapid detection of Lumpy Skin Disease Virus (LSDV) from cattle

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Abstract Lumpy skin disease virus (LSDV) is an infectious double-stranded DNA virus of the genus *Capripoxvirus* that causes Lumpy skin disease (LSD), a serious problem in cattle livestock. LSD has the potential to spread rapidly and cause substantial economic damage. A key to preventing the spread of LSDV in cattle is rapid and accurate detection. Loop-mediated isothermal amplification (LAMP) assay is a novel technique that works by amplifying the specific target nucleic acid at a constant temperature, which is simple, fast, and easy to use in the field. This study developed a prototype LAMP assay to detect LSDV from various cattle samples, as compared to the conventional PCR assay. The results showed that, out of the total 30 cattle sampled with clinical signs of LSDV, 16.67% were positive for LSDV when detected by PCR assay, while 36.67% were positive for LSDV when detected by LAMP assay, indicating that the LAMP assay had a higher accuracy than conventional PCR assay. Moreover, the results showed that the LAMP assay had the same sensitivity as the conventional PCR assay, and both assays had highest detection rates when detected from nasal swab samples. Additionally, the nucleotide alignment was 100% identical to the LSDV strains from China, Hong Kong, and Vietnam. The LAMP assay can effectively detect the GPCR gene in LSDV from cattle samples, and it can be further used for developing a LAMP test kit to detect LSDV in the field.

Keywords: Lumpy skin disease virus (LSDV), Lumpy skin disease (LSD), G-protein-coupled Chemokine receptor (GPCR), Loop-mediated isothermal amplification (LAMP)

Introduction

Lumpy Skin Disease (LSD) is an infectious disease in cattle that affects the epithelial tissues (Molla *et al*., 2017). It is a serious problem in cattle livestock around the world because it has the potential to spread rapidly, thus

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causing significant economic loss. Additionally, LSD poses a constraint to the livestock industry by making it difficult to export cattle or the product for sale due to the fever, reduction in milk production, weight loss, and damage to the hides (Woods, 1988; Bowden *et al*., 2008; Tuppurainen *et al.,* 2017; Bedeković *et al.,* 2018; Sprygin *et al*., 2018a). The LSD has a low mortality rate but high morbidity rate, and it affects cattle of all ages, sexes, and breeds (Badhy *et al.,* 2021). The clinical signs of LSD are high fever, reduced milk yield, lymphadenitis, and nodular skin lesions (size is 10-50 mm) (OIE, 2017; Namazi and Khodakaram Tafti, 2021).

LSD is caused by Lumpy Skin Disease Virus (LSDV), which is an infectious double-stranded DNA virus in the genus: *Capripoxvirus*, subfamily: *Chordopoxvirinae*, and family: *Poxviridae*, which is the same as the sheep pox virus (SPPV) that infects sheep, and goat pox virus (GTPV) that infects goat (Tulman *et al*., 2001). LSDV has a width of 262 ±22 nm and length of 294 ±20 nm and oval or brick shape (Kitching and Smale, 1986). The genome of LSDV is about 151 kilo base (kb) and encodes 156 putative genes, and over 97% identical to the genomes of other members of the genus *Capripoxvirus* (Tulman *et al.,* 2001; Tulman *et al.,* 2002). LSDV has a G-protein-coupled chemokine receptor (GPCR), which is an important gene for immunosuppression and proliferative lesions in the host cell. GPCR binds to the extracellular factors of the host cells, resulting in abnormal intracellular signal transduction and induces immunosuppression in the host (Lalani *et al.,* 1999; El-Tholoth and El-Kenawy, 2016). Currently, the GPCR gene is widely used for molecular detection of LSDV in cattle and used for studies of genetic variation of *Capripoxvirus* (Cao *et al.,* 1995; Le Goff *et al.,* 2009; El-Tholoth and El-Kenawy, 2016; Cêtre-Sossah *et al.,* 2017).

The first recognized extensive outbreak of LSD occurred in 1929 in Zambia (Woods, 1988). From 1943-1944, the LSD outbreak involved many countries in South Africa until the 1950s when it continued to spread throughout Africa, beginning in northern Sudan and then westward (Nawathe *et al*., 1982; Khalafalla *et al.,* 1993). Between 1988-1989, the first reported LSD outbreak outside of Africa was confirmed in Egypt and Israel (Brenner *et al*., 2006). Since 1989, cases of LSD have been reported in Bahrain, Kuwait, Oman, Yemen, Lebanon, Jordan, and many countries in Asia (Awad *et al.,* 2010; Gari *et al.,* 2012). From 2015-2018, it spread to Turkey, Greece, Balkans, Russia, and Ugandan (Agianniotaki *et al*., 2017; Şevik and Doğan, 2017; Mercier *et al.,* 2018; Sprygin *et al*., 2018; Ochwo *et al*., 2020). In 2019, LSD continued to spread throughout Asia, to China, India, Nepal, Bhutan, Taiwan, Laos and Thailand (Rozstalnyy *et al*., 2020; Hasib *et al*., 2021; Lu *et al*., 2020; Arjkumpa *et al*., 2022).

Currently, the detection of LSDV has relied on cell culture techniques and polymerase chain reaction (PCR), but these techniques still have limitations because they require high biosafety level, specialized personnel, costly equipment, and a long time to results. The rapid and accurate detection of LSDV in cattle is an important part of prevention of the spread of LSDV. Loop-mediated isothermal amplification (LAMP) is a molecular technique used for amplifying nucleic acid at a constant temperature, which is simple, fast, and easy to use in the field. LAMP assay has higher accuracy, specificity, and efficiency than conventional PCR (Notomi *et al.*, 2000). The DNA amplification in LAMP method uses a combination of four or six primers (Murray *et al*., 2013): forward inner primer (FIP; include two binding targest: F1c, and F2), backward inner primer (BIP; include two binding targets: B1c, and B2), forward outer primer (F3), and backward outer primer (B3). Six-primer reactions are optional for enhancing the sensitivity of the LAMP assay that consists of FIP, BIP, F3, B3, forward loop primer (Loop-F), and backward loop primer (Loop-B) (Amaresh *et al*., 2012). Each primer binds to a specific sequence in the target gene. Moreover, LAMP assay does not require specialized personnel, expensive equipment, or high biosafety level. Additionally, its time to result is typically less than 1 hour (Parida *et al.*, 2008). The LAMP results can be monitored in several ways without the need for complicated instrument. LAMP products can be monitored by running on an agarose gel electrophoresis, measuring the fluorescence using a DNA-intercalating dye in a real-time fluorescent detector, observing the turbidity of magnesium pyrophosphate in a spectrophotometer that is the by-product of LAMP reaction, and visualizing the fluorescence under UV light in a dark chamber (Notomi *et al*., 2000; Mori *et al.*, 2001; Parida *et al*., 2008). Moreover, LAMP assay does not require a thermocycler because LAMP reaction can amplify nucleic acid under a constant temperature, thus it is suitable and convenient for use in the field.

This study aimed to develop a LAMP assay prototype for rapid detection of LSDV from cattle samples with LSD in the field, in comparison to a conventional PCR assay, using samples from the 2021 outbreak in Asia.

Materials and methods

Sample collection

The LSDV samples were collected from cattle with clinical signs of LSD in Phetchaburi province, Thailand. Blood, nasal swab, saliva, and fecal samples were collected from a total of 30 cattle, following the standard operating

procedure of the Phetchaburi Provincial Livestock Office. All animal procedures in this study was reviewed and approved by Silpakorn University Animal Care and Use Committee (protocol number 17/2564). For each cattle, 3 ml of blood sample was collected from the jugular vein into a blood collection tube with Ethylenediaminetetraacetic acid (EDTA). The nasal swab sample was collected by inserting the sterile cotton swab into the nostril and rotating the cotton swab thoroughly inside of nostril, then storing cotton swab in a sterile tube. Saliva sample was collected by inserting the sterile cotton swab into the mouth and rotating thoroughly inside of mouth. Fecal sample was collected from the rectum and put into sterile plastic bag. After collection, all samples were placed on ice immediately and then transfered to the laboratory and kept at -20 ℃ until use.

DNA Extraction

All cattle samples were extracted for DNA using different protocols depending on the type of the samples. DNA extraction from blood, nasal swab, and saliva samples were performed using gSYNC™ DNA extraction kit (Geneaid, Taiwan) according to the manufacturer's recommendation. DNA extraction from fecal samples was done following the protocol developed by Zhang *et al*. (2006). All DNA samples were kept at -20 ℃ until use.

Detection of LSDV by PCR assay

Cattle DNA samples were used as templates for detecting the GPCR gene by PCR assay. The PCR primers were designed using the Geneious Prime program (Biomatters, NZ) specifically targeting the GPCR gene with the following sequences; forward primer: 5′-CCAATGCTAATACTACCAGCACTAC -3′ and reverse primer: 5′-CTTAGTACAGTTAGTAGCGCAACC -3′. The amplicon size of the PCR product is 1,130 base pair (bp). The final volume of 25 µl PCR mixture containing 2 µl of DNA template from cattle samples, 1 µl of forward primer, 1 µl of reward primer, 12.5 µl of OneTaq Hot Start master mix (New England Biolabs, USA), and 8.5 µl of ddH_2O. The following PCR condition was used: 94 ℃ for 3 min of initial denaturation; 34 cycles of 94 ℃ 30 seconds for denaturation, 50 ℃ 30 seconds for annealing, and 68 ℃ 45 seconds for extension; and a final extension at 68 ℃ 5 minutes. The PCR products were verified by running 1.0% agarose gel electrophoresis and visualized in a UV transilluminator. Representative PCR products were extracted and sequenced using the BTseq sequencing method (U2Bio, Thailand).

Detection of LSDV by LAMP assay

The DNA from cattle samples were detected at GPCR gene by LAMP assay using the LavaLAMP kit (Lucigen®, USA). The primers specifically targeting the GPCR gene were designed using PrimerExplorer V4 software (https://primerexplorer.jp/e/index.html) (Table 1). The final volume of 25 µl LAMP reaction mixture containing; 1 µl of the DNA template from cattle samples, 10 µM of target-specific primer, 0.5 µl of F3 and B3 primer, and 2 µl of FIP and BIP primer, 1 µl of green fluorescent dye, 12.5 µl of LavaLAMP™ DNA master mix, and 5.5 µl of ddH_2O. The LAMP amplification takes place under the condition of 70 ℃ for 30-60 minutes, and hold at 4 ℃ until detection. The LAMP products were measured for fluorescence using the FAM channel in a real-time fluorescent detector, visualized for fluorescence under UV light, measured for turbidity of magnesium pyrophosphate in a spectrophotometer in OD600, and run on 1.0% agarose gel electrophoresis and visualized in UV transilluminator

Table 1. Primers specifically targeting the GPCR gene used for LAMP assay

Gene		Primer sequence
GPCR	F3	5'- TGCTACGCAATCGTAAAAGC -3'
	B3	5'- TCAGTATTGTTTTTACTCCCATT -3'
	FIP	5'- AGCTGAAATTGTGTCTCTATGTCATTAGTAAATTCTCTACTACAAAACGC -3'
	BIP	5'- GAACTGCAAGGTTGACAAATCTTAAAGTGTAACTGTATTTGTTTCATCGT -3'

Sensitivity test

To determine the sensitivity of PCR and LAMP assay, genomic DNA from cattle sample was 10-fold serially diluted with the elution buffer and measured by nanodrop spectrophotometer (Eppendorf BioSpectrometer® basic, Germany) at the concentration of 48.5, 4.85, 0.49, 0.04, 0.004, 0.0004, 0.00004, and 0.000004 ng/µl. The DNA solution at each concentration was used as a template for PCR and LAMP assay.

Sequence analysis sequencing data of GPCR genes

The sequences of GPCR gene of cattle samples following PCR and BTseq sequencing were analyzed using the Geneious Prime program for nucleotides alignment with other sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The alignment output was used to calculate the sequence similarity score and to generate the neighbor-joining phylogenetic tree.

Results

Detection of LSDV by PCR assay

DNA samples from 30 cattle (C1-C30) was extracted and detected for GPCR gene by PCR assay. PCR products were separated on 1.0% agarose gel and showed amplification of GPCR gene in 5 samples (16.67%) consisting of C1, C4, C5, C19, and C22 (Table 2). Among the detected samples, nasal swab from C1, C4, C5, C19, and C22, and saliva sample from only C22 showed amplification of GPCR gene, while blood and fecal sample did not show amplification of GPCR gene. The results indicate that the samples C1, C4, C5, C19, and C22 were positive for LSDV.

Table 2. Detection of LSDV in GPCR gene from blood, fecal, nasal swab, and saliva samples by PCR assay

Sample	GPCR gene			
	Blood	Fecal	Nasal swab	Saliva
C1	-	-	+	-
C2	-	-	-	-
C3	-	-	-	-
C4	-	-	+	-
C5	-	-	+	-
C6	-	-	-	-
C7	-	-	-	-
C8	-	-	-	-
C9	-	-	-	-
C10	-	-	-	-
C11	-	-	-	-
C12	-	-	-	-
C13	-	-	-	-
C14	-	-	-	-
C15	-	-	-	-
C16	-	-	-	-
C17	-	-	-	-
C18	-	-	-	-
C19	-	-	+	-
C20	-	-	-	-
C21	-	-	-	-
C22	-	-	+	+
C23	-	-	-	-
C24	-	-	-	-
C25	-	-	-	-
C26	-	-	-	-
C27	-	-	-	-
C28	-	-	-	-
C29	-	-	-	-
C30	-	-	-	-

Detection of LSDV by LAMP assay

DNA samples that showed positive PCR results (PCR+) and negative PCR results (PCR-) were detected for GPCR gene using LAMP assay, along with LAMP positive control (DNA and primers control) and negative control (no-template control). The primers were designed specifically to target the GPCR gene (Figure 1). Detection of amplification reaction using fluorescent dye in FAM channel in real-time fluorescent detector showed increasing signal, indicating the positive amplification of GPCR gene in the PCR+ sample with the time to positive (Tp-value) at 16.62 minutes, while the lack of signal indicated a negative amplification of GPCR gene and no Tp-value in the PCR- sample (Figure 2). Confirmation of LAMP product using 1.0% agarose gel electrophoresis showed amplification of GPCR gene in PCR+. Because the LAMP products typically have various sizes of the DNA amplicons, the characteristic LAMP bands on the agarose gel showed laddering and smearing patterns (Figure 3). The results indicated that the LAMP assay can be used for detection of LSDV in GPCR gene from cattle samples.

Figure 1. LAMP primer design specifically targeting the GPCR gene in LSDV (GenBank: AF325528) consists of forward inner primer (FIP; includes two binding targets: F1c, and F2), backward inner primer (BIP; includes two binding targets: B1c, and B2), forward outer primer (F3), and backward outer primer (B3)

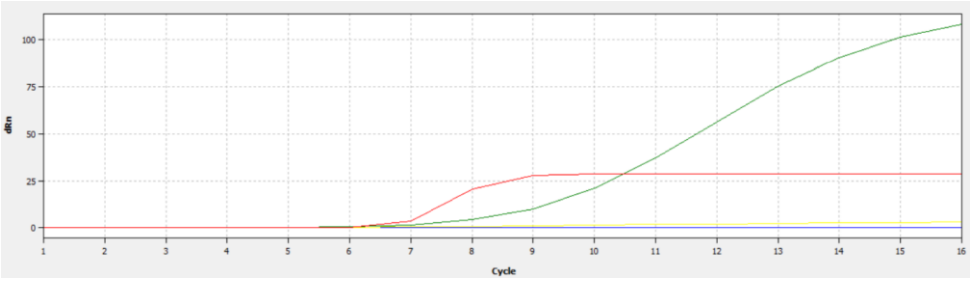

Figure 2. Analysis of the LAMP assay by measuring the fluorescence in a real-time detector using the FAM channel. Red line: positive control, blue line: no-template control, green line: PCR+, and yellow line: PCR-

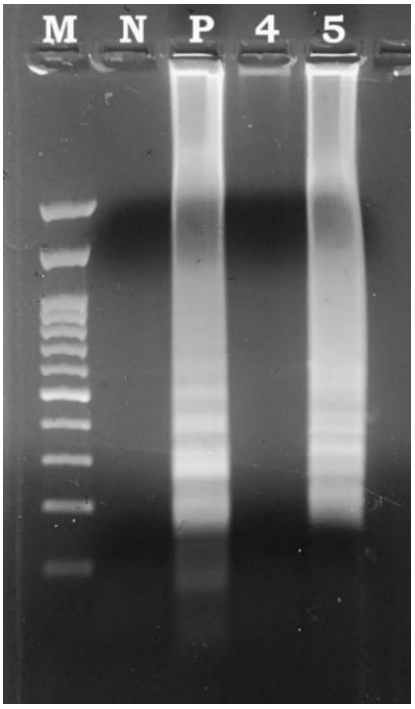

Figure 3. Analysis of the LAMP assay by running on a 1.0% agarose gel electrophoresis showing the amplification products from the GPCR gene. Lane M: 100 bp DNA ladder, lane N: no-template control, lane P: positive control, lane 4: PCR-, and lane 5: PCR+

Thirty nasal swab DNA samples (C1-C30) were used as templates for detection of LSDV by LAMP assay with LAMP positive control and negative control. The primers were designed specifically targeting the GPCR gene. The results were calculated by qPCRsoft program (Jena, Germany), which

calculates the appropriate cuttoff point of the positive and negative results. The samples detected using the real-time fluorescent assay (FAM channel) showed an amplification of GPCR gene in 11 samples (36.67%) consisting of C1, C4, C5, C6, C7, C9, C11, C13, C19, C22, and C26 (Table 3), in this case, the cutoff point is Tp = 30.

Table 3. Detection of LSDV using the GPCR gene from nasal swab samples with positive control and negative control by LAMP assay

Sample	GPCR gene	
	results	Tp-value (minutes)
Positive Control		15.18
No-template control		No Tp
C1	+	26.56
C2	-	No Tp
C3	-	31.80
C4	+	27.99
C5	+	25.68
C6	+	25.24
C7	+	20.83
C8	-	34.26
C9	+	24.67
C10	-	No Tp
C11	+	26.37
C12	-	No Tp
C13	+	23.68
C14	-	43.60
C15	-	No Tp
C16	-	43.83
C17	-	No Tp
C18	-	39.88
C19	+	29.13
C20	-	No Tp
C21	-	43.13
C22	+	23.98
C23	-	40.30
C24	-	34.56
C25	-	37.74
C26	+	23.60
C27	-	39.25
C28	-	No Tp
C29	-	38.09
C30	-	38.21

Detection of LSDV in different sample types

Different types of cattle samples consisting of blood, nasal swab, saliva, and fecal samples were detected for the GPCR gene using PCR and LAMP assays. The PCR products were analyzed on a 1.0% agarose gel electrophoresis, which showed the highest detection rate in the nasal swab samples (16.67%), and saliva samples (3.33%), but no amplification from blood and fecal samples (Table 4). In one particular case, the LAMP assay was used to detect the GPCR gene from different types of cattle samples including blood, nasal swab, saliva, and fecal sample from the same cattle (C22), and LAMP results showed detected LSDV in only the nasal swab sample but no amplification from blood, saliva, and fecal samples (Figure 4).

Table 4. The detection rate of different types of cattle samples using PCR assay

Sample	Detection rate
Blood	0%
Nasal swab	16.67%
Saliva	3.33%
Fecal	0%

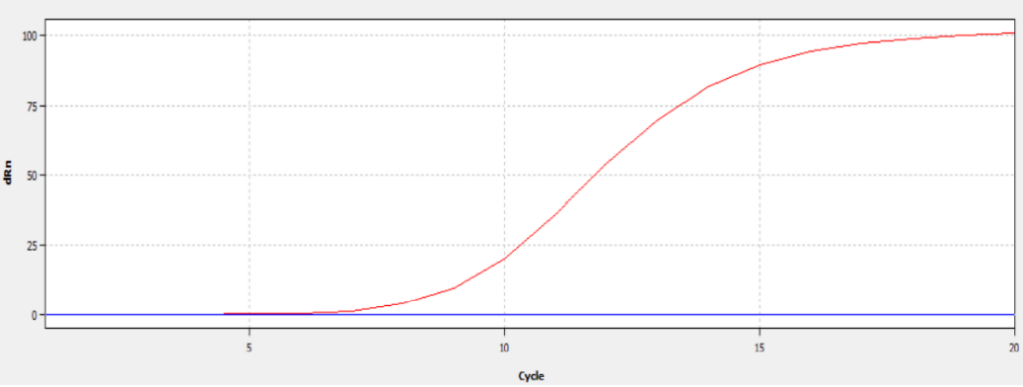

Figure 4. Analysis of LAMP assay by fluorescent assay in real-time fluorescent detector in the FAM channel in different types of cattle samples. Upper line (red): nasal swab sample, lower line (blue): blood, saliva, and fecal sample (no amplification)

Sensitivity test

The LSDV-positive DNA was 10-fold serially diluted and used as templates to determine the sensitivity of PCR and LAMP assay for detecting the GPCR gene. The concentration of DNA in each dilution tube was measured by nanodrop spectrophotometer (Eppendorf BioSpectrometer® basic, Germany) consisting of 48.5, 4.85, 0.49, 0.04, 0.004, 0.0004, 0.00004, and 0.000004 ng/µl. The results showed the lowest limit of detection at the concentration of DNA template in both PCR and LAMP reaction at 0.04 ng/µl (Figure 5). This indicates that the sensitivity of LAMP assay and PCR assay are similar.

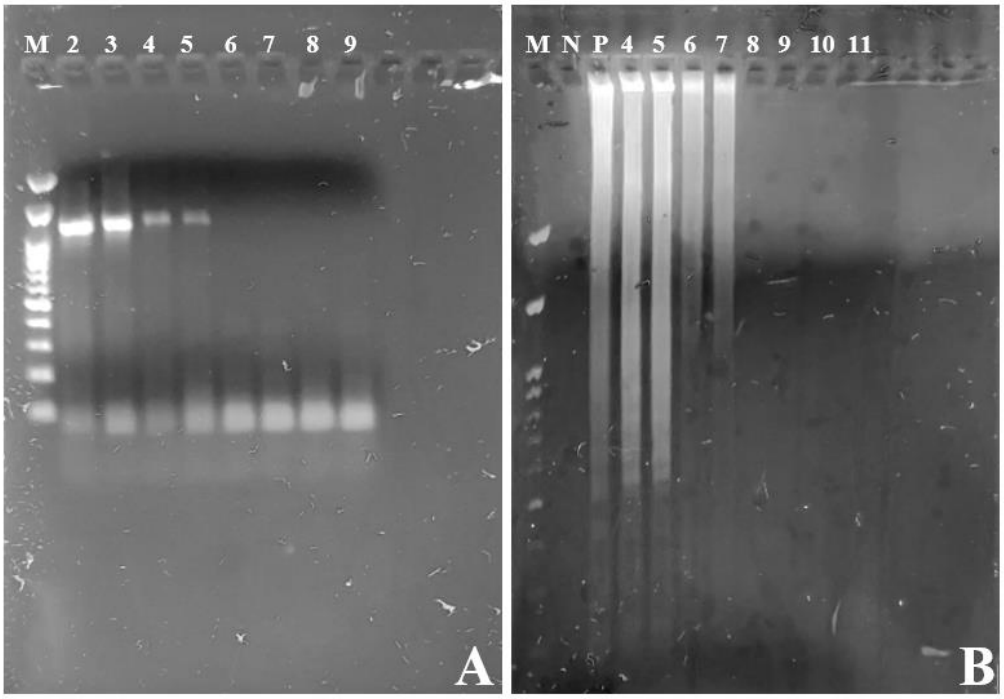

Figure 5. Sensitivity test of PCR and LAMP assay in GPCR gene on 1.0% agarose gel electrophoresis. (A) PCR assay; lane M: 100 bp DNA ladder, lane 2: original DNA concentration 48.50 ng/µl, lanes 3-9: ten-fold serially-diluted DNA at 4.85, 0.49, 0.04, 0.004, 0.0004, 0.00004, and 0.000004 ng/µl respectively. (B) LAMP assay; lane M: 100 bp DNA ladder, lane N: no-template control, lane P: positive control, lane 4: original DNA concentration 48.50 ng/µl, lanes 5-11: ten-fold serially diluted DNA at 4.85, 0.49, 0.04, 0.004, 0.0004, 0.00004, and 0.000004 ng/µl respectively

Sequence analysis of the GPCR gene

The PCR product from the nasal swab sample (C22) that showed positive amplification of the GPCR gene (amplicon size 1,066 bp) was purified for sequencing using BTseq (U2Bio, Thailand) (Figure 6), and analyzed using the Geneious Prime program for nucleotide alignment (Figure 7) and neighbor-joining phylogenetic analysis (Figure 8). The amplified GPCR sequence in this study was compared with the GPCR gene sequences of other LSDV, GTPV, and SPPV sequences available on GenBank. The GPCR gene of LSDV (C22) sample showed identical nucleotide sequence with LSDV MW251475 (China, 2021), MW355944 (China, 2020), MW732649 (Hong Kong, 2020), MZ577073 (Vietnam, 2021), MZ577074 (Vietnam, 2021), and MZ577075 (Vietnam, 2021) (100% identity), and similar nucleotide sequences with the Neethling strain 2490 AF325528 (Africa) (99.2% identity), and comparison with other viruses in the genus *Capripoxvirus* consisting of KF661979 (SPPV, China,2013), and KF495252 (GTPV, India, 2015) showed 95.6% and 96.4% identity respectively (Table 5).

1	ACTACAATTATTAGCACAATTCTCAGTACAATTTCAACAAATCAAAATAATGTTACAACGCCTTCAACTTATGAAAATACAACAACGATATCTAATTATA	100
101	CAACCGCATATAATACAACTTATTATAGCGATGATTATGATGATTATGAAGTGAGCATAGTCGATATCCCACATTGTGATGATGGTGTGGATACTACAAG	200
201	TTTTGGACTGATTACTTTATATTCGACTATATTCTTTCTTGGATTATTTGGAAATATAATTGTGTTAACTGTTCTTCGTAAATATAAGATAAAAACAATA	300
301	CAGGATATGTTTTTGCTTAATTTGACACTGTCTGATTTAATTTTCGTGTTGGTGTTTCCTTTTAATTTATACGATAGTATCGCTAAACAATGGAGTTTAG	400
401	GAGATTGTTTGTGTAAATTTAAAGCTATGTTTTACTTTGTTGGTTTTTACAATAGCATGTCATTTATAACATTGATGAGTATTGATAGATACCTAGCTGT	500
501	AGTTCACCCAGTAAAATCAATGCCGATAAGGACAAAACGATATGGAATTGTACTTAGTATGGTGGTTTGGATTGTCTCAACTATTGAATCCTTTCCAATA	600
601	ATGTTATTTTATGAAACAAAAAAAGTATATGGAATAACGTATTGTCATGTATTTTATAACGATAATGCAAAAATTTGGAAATTATTTATAAATTTTGAAA	700
701	TAAACATATTTGGAATGATTATACCGCTAACTATTTTGCTATATTGTTATTATAAAATCTTAAATACTTTAAAAACCTCGCAAACAAAGAATAAGAAAGC	800
801	CATAAAGATGGTGTTTTTGATTGTTATCTGTTCAGTATTGTTTTTACTCCCATTTAGTGTAACTGTATTTGTTTCATCGTTGTATTTGTTAAATGTTTTT	900
901	AGTGGATGTACGGCATTACGATTTGTCAACCTTGCAGTTCATGTAGCTGAAATTGTGTCTCTATGTCATTGTTTTATTAATCCACTAATTTATGCGTTTT	1,000
1,001	GTAGTAGAGAATTTACTAAAAAGCTTTTACGATTGCGTAGCACTAGTAGTGCTGGTAGTATTAGCA	1,066

Figure 6. Nucleotide sequences of GPCR gene from cattle nasal swab sample (C22)

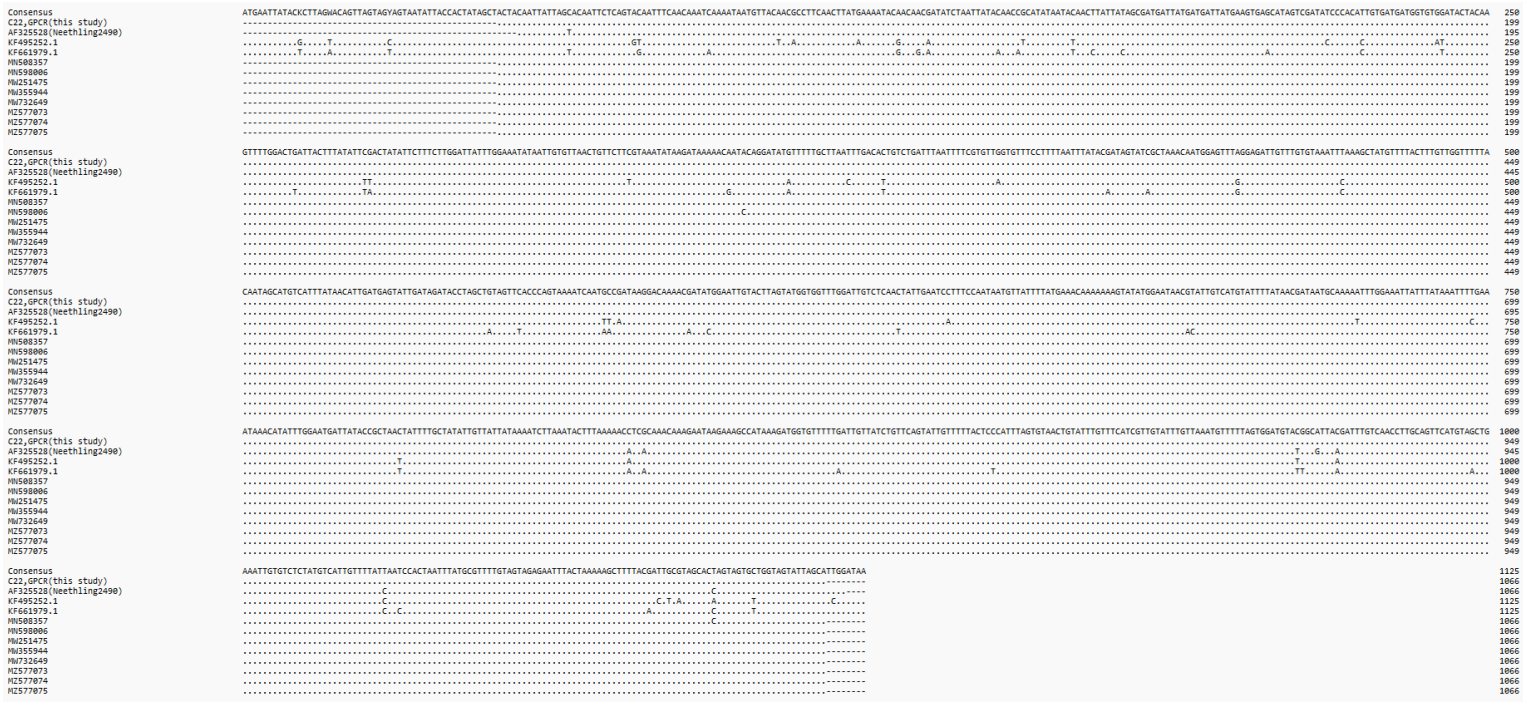

Figure 7. Nucleotide alignment of GPCR gene in cattle nasal swab sample (C22) with the GPCR gene sequences of LSDV, GTPV, and SPPV available on GenBank

Table 5. Identity analysis of the GPCR gene in cattle nasal swab sample (C22) with the GPCR gene sequences of LSDV, GTPV, and SPPV available on GenBank

Sequence	AF325528	C22, GPCR	MN508357	MN598006	MW251475	MW355944	MW732649	MZ577073	MZ577074	MZ577075	KF495252	KF661979
AF325528 (Neethling2490)												
C22, GPCR (this study)	99.2											
MN508357 (China, 2019)	99.3	99.9										
MN598006 (Xinjiang, 2019)	99.2	99.9	99.8									
MW251475 (China, 2021)	99.2	100.0	99.9	99.9								
MW355944 (China, 2020)	99.2	100.0	99.9	99.9	100.0							
MW732649 (HongKong, 2020)	99.2	100.0	99.9	99.9	100.0	100.0						
MZ577073 (Vietnam, 2021)	99.2	100.0	99.9	99.9	100.0	100.0	100.0					
MZ577074 (Vietnam, 2021)	99.2	100.0	99.9	99.9	100.0	100.0	100.0	100.0				
MZ577075 (Vietnam, 2021)	99.2	100.0	99.9	99.9	100.0	100.0	100.0	100.0	100.0			
KF495252 (GTPV, India, 2015)	96.4	96.4	96.4	96.3	96.4	96.4	96.4	96.4	96.4	96.4		
KF661979 (SPPV, China, 2013)	96.2	95.6	95.7	95.5	95.6	95.5	95.6	95.5	95.6	95.5	95.4	

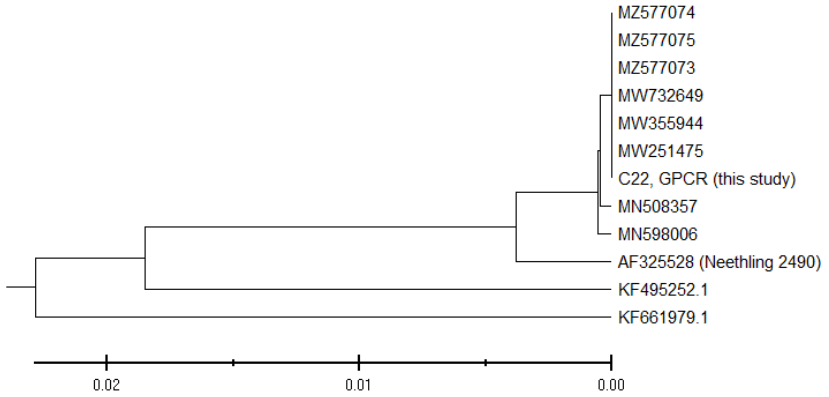

Figure 8. The neighbor-joining phylogenetic analyses of GPCR gene in cattle nasal swab sample (C22) with the GPCR gene sequences of LSDV, GTPV, and SPPV available in the GenBank

Discussion

The LSD is an economically devastating infectious disease in cattle livestock, and the outbreaks cause substantial economic losses for cattle livestock and the cattle industry worldwide (Bamouh *et al.,* 2021). It has the potential to spread rapidly between cattle via arthropod vectors such as mosquitoes (*Culex mirificens* and *Aedes natrionus*), ticks (*Riphicephalus appendiculatus* and *Amblyomma hebraeum*), and biting flies (*Stomoxys calcitrans* and *Biomyia fasciata*) (OIE, 2017). Moreover, LSD can be transmitted by infected semen, milk, and placenta (Tuppurainen, 2015). However, direct contact in cattle can also be a minor source of LSD transmission (Hanshaw *et al.,* 1968; El-Kholy *et al*., 2008). Rapid and accurate detection is an important key to preventing the spread of LSD in cattle livestock.

Detection of LSDV is commonly done by PCR assay on DNA extracted from cattle samples, using primers targeting the GPCR gene that controls the immunosuppression and proliferative lesions in the host cell (El-Tholoth and El-Kenawy, 2016). Currently, the GPCR gene is widely used for molecular detection of LSDV in cattle and buffalo and for studies of genetic variation of *Capripoxvirus* consisting of LSDV, GTPV, and SPPV (Cao *et al.,* 1995; Le Goff *et al*., 2009; El-Tholoth and El-Kenawy, 2016; Cêtre-Sossah *et al*., 2017). A previous report shows a high rate of LSDV detection using the skin samples from skin lesions (Awad *et al*., 2010), although the collection of skin sample from cattle is considerably invasive. In this study, the rate of LSDV detection using PCR assay showed amplification of GPCR gene at 16.67% (5/30 cattle). Nasal swab samples showed the highest detection rate at 16.67% (5/30), while saliva samples showed the detection rate at 3.33% (1/30). Blood and fecal samples did not show amplification of GPCR gene. This indicates that nasal swab sample collection can be used as a non-invasive method for detection of LSDV in cattle. All cattle showed some clinical signs of LSD at the time of sample collection, but the result in this study indicated that the PCR assay could only detect the infection from some of the cattle.

For the LAMP assay, detection of LSDV from cattle nasal swab samples showed the amplification of GPCR gene at 36.37% (11/30), which is higher than the PCR assay. In this study, all PCR-positive samples were also LAMP-positive. However, some LAMP-positive samples were not detected as positive in the PCR assay. The LAMP results indicated that the LAMP assay had a higher detection rate and accuracy than conventional PCR assay, most likely because the LAMP assay used 4 primers that were specific to LSDV, which resulted in a higher accuracy. Moreover, the PCR results were monitored by a 1.0% agarose gel electrophoresis that indicated either a positive or negative result, while the LAMP results were monitored by fluorescent assay using a

real-time fluorescent detector that can detect quantitative signals at a lower threshold level. The results, visualization of turbidity of magnesium pyrophosphate, showed that all sample tubes had similar turbidity. Measurement of the optical density at 600 nm by a spectrophotometer showed that the sample that were LAMP-positive in the fluorescent assay also showed higher OD than the samples that were negative in the fluorescent assay. Visualization of end-point products of LAMP under UV light showed that all sample tubes were slightly different. The positive tube in the fluorescent assay in FAM channel from the real-time fluorescent detector had a higher fluorescence intensity than the negative tubes, which is likely caused by the high efficiency of the green fluorescent dye in the reaction. When nucleotide or primer is bound, the fluorescent glow occurs under the UV light narly immidiately. Therefore, the visualization of turbidity and visual fluorescent were in accordacne with the fluorescent assay, but visualization of turbidity and fluorescent were difficult to judge visually.

Using different types of samples for the detection of LSDV resulted in different detection rates. We performed DNA extraction using different protocols depending on the type of the samples. DNA extraction from blood, nasal swab, and saliva samples was performed using the gSYNC™ DNA extraction kit (Geneaid, Taiwan). In this study, the LSDV can be detected in 5 samples of nasal swab and 1 sample of the saliva, but not detected in blood, which indicated that the DNA extraction method from blood, nasal swab, and saliva did not affect the detection rate. Extraction from fecal samples was done using Zhang *et al*. (2006) protocol, which started with fecal consisting of water, bacterial biomass, proteins, fats, fibers, and inorganic substances (Lukas *et al.*, 2005). The fecal DNA extraction protocol does not utilize a silica column for separation of DNA and other components, which may cause interferance or disruption of the PCR and LAMP reaction.

All cattle samples were collected from cattle with clinical signs of LSD such as high fever (>40.50 ℃), malaise, anorexia, lymphadenitis, and nodular skin lesions. In this study, the PCR and LAMP results showed low detection rate. This may be due to the fact that farmers in Phetchaburi province, Thailand did not clearly record the symptom and duration of infection of LSD from the cattle on their farm, thus, some cattle may already be in the last stage of LSD before recovering. Additionally, some cattle are almost cured from LSD but still have skin lesions, making the molecular detection difficult. It is known that the LSDV targets epithelial cells such as nodular skin lesions, which may have caused the low detection rate in this study (Awad *et al.,* 2010). However, LSD generally had a low mortality rate (1-2%) (MacLachlan and Dubovi, 2011), and most infected cattle can recovered without any intervention. Moreover, some symptom such as fever, weakness, malaise, and anorexia are caused by

secondary infections and lameness (Tuppurainen, 2015), which can cause the LSDV negative detection results as well.

The sensitivity test results between PCR and LAMP assay were nearly identical. Depending on the primer design in each assay that targets the GPCR gene. Both PCR and LAMP assay can be used for detection of the LSDV in cattle samples with a low concentration of DNA (down to 0.04 ng/µl). As a result, the LAMP assay had the same sensitivity as the conventional PCR assay, so the LAMP assay can be further developed into a rapid detection test kit that can be used in the field.

The PCR products were obtained from amplification of GPCR gene and analyzed by 1.0% agarose gel electrophoresis, followed by sequencing of the GPCR gene. The sample from Phetchaburi province, Thailand has 100% identical nucleotide sequence similarity to LSDV in China, Hong Kong, and Vietnam. The outbreak in Phetchaburi province, Thailand was probably caused by an import of infected cattle from another country, which caused the nucleotide sequence to be the same as in China, Hong Kong, and Vietnam. A recent report in 2021 showed that the genome of LSDV Hong Kong strain (MW732649) had 99.2% identity with the Neethling strain 2490 (Africa), which is consistant with this study (Flannery *et al*., 2021). Moreover, GPCR gene in this study had a small difference of nucleotide sequences with the Neethling strain 2490 (Africa), which is the origin country during the outbreak of LSD in 1929-1988 from Africa. Since then, the disease spread to many countries for a long time resulting in the genetic mutation of LSDV throughout the outbreak period, until LSDV spread to Phetchaburi province in Thailand. The outbreak of LSDV in Phetchaburi province, Thailand was likely not caused by a live-attenuated virus vaccination, because the Neethling-type vaccine (MSD Animal Health) was primarily used in Thailand since the beginning of the latest outbreak. If the outbreak in Phetchaburi provinces is caused by vaccination, the nucleotide sequences of LSDV should have been most similar to that of the Neethling strain from Africa, and not the China/Hong Kong/Vietnam strain.

Finally, the LAMP assay was able to amplify the GPCR gene of LSDV from cattle samples, although a caution must be taken to ensure the correct infection stages and specific primer design. The LAMP assay can be used as a prototype for developing a rapid detection test kit for diagnosis of LSDV in the field in the future.

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